

Published in final edited form as:

J Thromb Thrombolysis. 2012 April ; 33(3): 246–257. doi:10.1007/s11239-012-0683-0.

Time-dependent changes in non-COX-1-dependent platelet function with daily aspirin therapy

Deepak Voora, MD, Thomas L. Ortel, MD, PhD, Joseph E. Lucas, PhD, Jen-Tsan Chi, MD, PhD, Richard C. Becker, MD, and Geoffrey S. Ginsburg, MD, PhD

Abstract

Objectives—To develop an integrated metric of non COX-1 dependent platelet function (NCDPF) to measure the temporal response to aspirin in healthy volunteers and diabetics.

Background—NCDPF on aspirin demonstrates wide variability, despite suppression of COX-1. Although a variety of NCDPF assays are available, no standard exists and their reproducibility is not established.

Methods—We administered 325mg/day aspirin to two cohorts of volunteers (HV1, n = 52, and HV2, n = 96) and diabetics (DM, n = 74) and measured NCDPF using epinephrine, collagen, and ADP aggregometry and PFA100 (collagen/epi) before (Pre), after one dose (Post), and after several weeks (Final). COX-1 activity was assessed with arachidonic acid aggregometry (AAA). The primary outcome of the study, the platelet function score (PFS), was derived from a principal components analysis of NCDPF measures.

Results—The PFS strongly correlated with each measure of NCDPF in each cohort. After two or four weeks of daily aspirin the Final PFS strongly correlated ($r > 0.7$, $p < 0.0001$) and was higher ($p < 0.01$) than the Post PFS. The magnitude and direction of the change in PFS (Final - Post) in an individual subject was moderately inversely proportional to the Post PFS in HV1 ($r = -0.45$), HV2 ($r = -0.54$), DM ($r = -0.68$), $p < 0.0001$ for all. AAA remained suppressed during aspirin therapy.

Conclusions—The PFS summarizes multiple measures of NCDPF. Despite suppression of COX-1 activity, NCDPF during aspirin therapy is predictably dynamic: those with heightened NCDPF continue to decline whereas those with low/normal NCDPF return to pre-aspirin levels over time.

Keywords

aspirin; platelets; light transmittance aggregometry; PFA100; principal components analysis

Introduction

Aspirin is the most commonly prescribed medication for the prevention of cardiovascular disease.[1] In both research and clinical settings the response to aspirin is measured using *ex vivo* platelet function testing.[2] Although widely used, there are limitations to this approach to measure an inhibitory response to a common drug like aspirin.[2] First, although variety of assays is available and light transmittance aggregometry (LTA) is the most commonly used platform, the optimal platform has not been defined.[2] Second, various stimuli can be employed; however, the optimal agonist or concentration has not been determined.[2] Finally, although a heightened aggregatory response to agonists, including epinephrine,

ADP, or collagen is a reproducible trait[3], prior studies of the consistency of the response to aspirin response over time have been limited to small sample sizes[4], retrospective analyses of aggregation data collected during clinical care[5] or were focused on platelet COX-1 activity[6]. Strategies to overcome these uncertainties must be resolved if *ex vivo* assays are to be used in clinical practice. Therefore, we sought to employ an integrated measurement of platelet function to measure the response to aspirin and investigate temporal changes in platelet function.

Aspirin is a potent inhibitor of COX-1 and an inhibitor of platelet function. When assays that are entirely dependent on platelet COX-1 are used (i.e., COX-1 dependent platelet function assays such as arachidonic acid aggregation [AAA] and serum thromboxane B2), there is minimal variation and near complete suppression of platelet function using 81–325mg aspirin doses.[7,8] Therefore, although such COX-1 dependent tests may best represent the biochemical response to aspirin, these may not provide a complete assessment of platelet function on aspirin since the production of thromboxane is but one pathway involved in platelet activation.[8]

Agonists such as collagen, epinephrine, and ADP can also stimulate platelet function through the generation of thromboxane. However, these assays are characterized by wide interindividual variability despite complete suppression of COX-1 activity[7,8] with aspirin and are independently associated with cardiovascular events in patients taking aspirin[9]. Therefore, these non COX-1 dependent platelet function (NCDPF) assays are important measures of the response to aspirin. However, no single agonist is sufficient to describe the platelet function response to aspirin, because, *in vivo*, platelets are presented with multiple stimuli. Therefore, for researchers studying platelet function or clinicians interested in the response to aspirin therapy it would be desirable to develop a single metric that integrates *multiple* NCDPF test results. Further, such an integrative metric may overcome some of the non-biologic variability associated with platelet function testing.

With the goal of developing a *single* metric that integrates multiple non-COX1 dependent platelet function (NCDPF) test results, we employed a principal components analysis to NCDPF data from an aspirin challenge study in healthy volunteers to derive a summary metric of platelet function that we term the platelet function score (PFS) and validated the PFS in independent cohorts of healthy volunteers and diabetics. To demonstrate the utility of the PFS, we 1) compared COX-1 vs. non-COX-1 dependent measures of platelet function and 2) investigated the reproducibility and time-dependent effects of NCDPF during daily aspirin therapy.

Methods

Aspirin challenge study -- overview

The Duke Clinical Research Unit (DCRU) and the Duke Institute for Genome Sciences & Policy (IGSP) coordinated several aspirin challenge studies from February 2009 to May 2011. The purpose of these ongoing studies is to examine gene expression profiles in healthy volunteers and diabetics at the extremes of platelet function in response to aspirin. The study design and sample sizes were therefore chosen for the purposes of analyzing gene expression data, not platelet function in response to aspirin *per se*. Nevertheless, this report represents a summary of the platelet function observations made as a part of these ongoing studies. An overview of the different cohorts and time points are described in Figure 1. We began with a pilot study of healthy volunteers (HV1). After the completion of this pilot study, we amended our study protocol for two subsequent studies in healthy volunteers and diabetics (HV2 and DM): increased duration of aspirin exposure, increased age requirement,

addition of arachidonic acid aggregation with and without *in vitro* aspirin, and a focus on those at the extremes of platelet function response.

For this study, the HV2 and DM cohorts serve as independent validation cohorts for the observations made in HV1.

From February through May 2009 a group of healthy adult volunteers (HV1) were recruited through advertisements and had a defined set of platelet function measurements (described below) made before aspirin (Pre), and 3-hours after (Post) a single 325mg tablet of aspirin. Subsequently, subjects were returned for a final assessment (Final) 2 weeks after 325mg/day aspirin. This dose of aspirin was chosen to ensure complete suppression of platelet COX-1 activity in each participant.[8]The 3-hour timepoint was chosen because we were interested in measuring the maximal inhibitory effect of aspirin[6].

The platelet function score (PFS, described below) was derived from a principal components analysis (PCA) of all platelet function measurements made in the HV1 cohort. From November 2009 through July 2011 we expanded our studies to diabetics, however in order to have a group of healthy volunteers whose age was similar to those of diabetics, we increased the age restriction to 30 years. This second group of healthy volunteers (HV2) and diabetics (DM) were recruited and assessed with an identical set platelet function measures made as in HV1 before (Pre) and 3-hours after (Post) a 325mg aspirin dose. Using their platelet function data we then calculated a PFS for each subject. The first 10 subjects in HV2 and DM were used to define the PFS distribution for each cohort, were selected to continue with 4 weeks of aspirin therapy, and returned for a final measurement of platelet function (Final). For subsequent subjects in the HV2 and DM cohorts, we selected those in the 1st and 4th quartile of their respective PFS distribution for additional aspirin therapy and final platelet function testing (Final).

Inclusion and exclusion criteria

This study included healthy volunteers greater than 18 (HV1) or 30 (HV2) years old; diabetics (defined by chart review and/or use of insulin or oral agent) must have been greater than 30 years of age. The following exclusion criteria applied to all cohorts: history of a bleeding disorder, gastrointestinal bleeding, regular use of antiplatelet agents (except aspirin in DM), nonsteroidal anti-inflammatory agents (NSAIDs), oral corticosteroids, anticoagulants, coexisting conditions: diabetes (except for HV1 and HV2), coronary artery disease, peripheral artery disease, cerebrovascular disease, history of stroke, deep venous thrombosis, transient ischemic attack, daily use of more than 1 prescription medication (for HV1 and HV2, except oral contraceptives and antihistamines), regular cigarette use (defined as > 1 cigarette/day), or known pregnancy.

Throughout the study period subjects were reminded to refrain from any new medications (in particular those containing aspirin or NSAIDs) and cigarette use. Subjects were given a list of over the counter medications that contain aspirin/NSAIDs to avoid. Dietary supplements were not an exclusion criteria and were not recorded, but instead, subjects were instructed to not alter their intake of any supplements throughout the study period. All study participants provided informed consent. The study protocol was approved by Duke University's Institutional Review Board.

Aspirin and NSAID washout

For subjects taking aspirin or NSAIDs prior to entry into the study, the subject was asked to reschedule their visit after at least 14 days of documented aspirin/NSAID abstinence.

Aspirin therapy

Medication adherence was a priority and subjects were required to record the date and time of each aspirin dose. Adherence was confirmed with a pill count at the end of the study. Finally, subjects received telephone reminders during the study to ensure adherence. Subjects that missed any of the three doses prior to the Final visit were given additional aspirin and rescheduled until adherence was established.

Platelet function studies

Before each visit, subjects were asked to fast and to refrain from tobacco (during the preceding 24 hours) and alcohol or intensive exercise on the day of testing. Phlebotomy was performed after 10 minutes of resting supine with minimal trauma or stasis at the venipuncture site using a 21-gauge needle into 3.2% sodium citrate tubes. The focus of these studies was around NCDPF and thus, we defined the NCDPF assays as the following: PFA100, and epinephrine, ADP, and collagen induced LTA. COX-1 dependent platelet function was initially not measured in HV1, however after observing the changes in NCDPF in HV1, we added COX-1 dependent measures to HV2. We chose serum thromboxane B2 and AA induced aggregation as measures of COX-1 activity and also added *in vitro* aspirin to the AA aggregation assay to further assess for any evidence of unsuppressed COX-1 activity.

Light transmittance aggregometry—Light transmittance aggregometry (LTA) was performed according to the method of Born[10] and the following agonists: arachidonic acid (AA, Chrono-log, 0.5 mM), epinephrine (Chrono-Log, 10, 1, and 0.5 μ M), ADP (Chrono-Log, 10, 5, and 1 μ M), and collagen (Chrono-Log, 5 and 2 μ g/ml). We chose the area under the LTA curve (AUC) as the primary measure of aggregation because the AUC captures several features of the aggregometry curve that are each sensitive to the effects of aspirin: slope[11,12], maximal aggregation[13], and final aggregation[14]. To standardize AUC measurements across individuals and visits we fixed the test duration at 12 minutes for epinephrine and six minutes for collagen, AA, and ADP. Further description of the AUC measurement is in the Supplemental Material.

Platelet Function Analyzer—PFA100 closure time with the use of the collagen/epinephrine cartridge was performed as previously described by our laboratory.[15] We did not select the collagen/ADP cartridge because its results are not sensitive to the effects of aspirin.[16] Briefly, citrated whole blood was placed in the PFA100 chamber and blood was aspirated through an aperture in a collagen/epinephrine coated membrane. The amount of time (in seconds[s], up to a maximum of 300 s) until blood ceased to flow through the membrane aperture was recorded as the final result.

Serum Thromboxane B2—Serum thromboxane B2 was measured in HV2 at the Final visit according to the method of Patrono[17]. Briefly, serum was collected by allowing whole blood to clot at 37 degrees Celsius for 45 minutes then centrifuged at 3000 rpm for 10 minutes. Serum was aspirated and stored at –80 degrees Celsius until ELISA testing. Thromboxane B2 measurements were performed using an ELISA based assay, in duplicate, averaged, and according to the manufacture's protocol (Enzo Life Sciences, Catalog No. ADI-900-002) with no modifications.

***In vitro* aspirin**—A stock solution of aspirin was created by dissolving acetylsalicylic acid (Sigma-Aldrich A5376) into DMSO and aliquots stored at –80 degrees Celsius. On the day of use, fresh aliquots were thawed, diluted in PBS, and stored at 4 degrees Celsius. This diluted aspirin solution was added to PRP to a final concentration of aspirin (53 μ M) that exceeds that achieved *in vivo* with a 325mg aspirin dose[18] or required to maximally

inhibit arachidonic induced aggregation[19]. The PRP was allowed to incubate with *in vitro* aspirin for 10 minutes at room temperature prior to the addition of agonist.

Statistical Analysis

Correlations between the various measures of platelet function were assessed with the Spearman correlation coefficient (r). Paired or unpaired t-tests were employed to compare continuous variables between groups and the Wilcoxon signed rank test was used for variables that were not normally distributed. Chi square tests were used to compare categorical variables between groups.

Missing Data—A small number of time-points ($n = 21$, 5%) in the collagen LTA data were missing and were imputed in the following manner: 1) Replace the missing values by the average for that assay. 2) Compute principal components. Let x be the $N \times P$ -dimensional matrix of platelet function measurements, where N is the number of samples and P is the number of different platelet function measurements. Compute the singular value decomposition: $x = UDV'$. We then compute D^* by setting to zero all but the first three diagonal elements of D . 3) Compute $x^* = UD^*V'$ and replace all missing values of x with the corresponding values from x^* . 4) Repeat steps 2 and 3 until the algorithm converges.

Principal Components Analysis—Our main interest was in studying NCDPF since others have shown minimal variation in COX-1 dependent platelet function measures on aspirin.[13] Because we observed linear relationships between the multiple measures of NCDPF (Table 2), we assumed that each measure of NCDPF was a reflection of a single biological factor and thus chose a principal components analysis (PCA) to quantify this biological factor. PCA is an unbiased, mathematical technique for reducing a collection of measurements down to a single “meta-measurement” that best describes the aggregate behavior of the set. For example, in the biological sciences, PCA can quantify population substructure in genome wide association studies[20], patterns of gene expression in microarray experiments[21], and metabolic pathways in metabolomic studies[22]. In the present study, the input into the PCA was the following set of non-imputed measures from the HV1 cohort: the PFA100 closure time and the AUC from each concentration of ADP, epinephrine, and collagen LTA. Each measurement was given equal weight and we defined the first principal component, which we constructed to be positively correlated with higher NCDPF, as the platelet function score (PFS).

To calculate PFS in a new sample we applied the weights from the PCA performed in HV1 and the new platelet function measurements (i.e., in HV2, DM, and imputed HV1 data) as follows:

$$\begin{aligned} \text{PFS} = & -(((\text{PFA100} - 199)/82 * 0.291) + ((\text{AUCADP10} - 296.87)/40.58 * - \\ & 0.349) + ((\text{AUCADP5} - 258.02)/56.75 * - 0.3398) + ((\text{AUCADP1} - 47.0698)/49.863 * - \\ & 0.24477) + ((\text{AUCEPI0}_5 - 120.0762)/137.58 * - 0.299) + ((\text{AUCEPI1} - \\ & 185.465)/173.75 * - 0.3462) + ((\text{AUCEPI10} - 343.576)/217.355 * - \\ & 0.387051) + ((\text{AUCCOL5} - 256.41)/76.85 * - 0.3579) + ((\text{AUCCOL2} - 193.84)/101.933 * - \\ & 0.3619)) \end{aligned}$$

Where, PFA100 = PFA100 collagen/epinephrine closure time (in seconds); AUCADP10, AUCADP5, and AUCADP1 = areas under the curve for ADP at 10, 5, and 1 μM , respectively; AUCEPI10, AUCEPI1, and AUCEPI0_5 = areas under the curve for Epinephrine at 10, 1, and 0.5 μM , respectively; AUCCOL5 and AUCCOL2 = areas under the curve for Collage at 5 and 2 mg/ml, respectively.

All analyses were performed in R (version 2.8.1). All statistical tests were two-sided and a p-value < 0.05 was considered significant.

Results

The baseline characteristics of the three cohorts are described in Table 1. Besides differences in medications, age was the only significantly different baseline characteristic between cohorts. As outlined in Figure 1, pre-aspirin (Pre) and the 3 hour post-aspirin (Post) measurements were made in 52 subjects in HV1, 96 in HV2, and 74 in DM. Per our selection protocols (described in the Methods), the following numbers of subjects returned for the Final visit in each cohort: 52 in HV1, 52 in HV2, and 42 in DM.

Construction of the platelet function score (PFS) as a surrogate for non COX1 dependent platelet function (NCDPF)

We observed strong and significant correlation between various measures of NCDPF made before and after aspirin exposure in HV1 (Table 2). To condense these measures of platelet function into a single metric we conducted a PCA on the PFA100 closure time and the AUCs induced by epinephrine, ADP, and collagen to derive the PFS on all timepoints in HV1. As anticipated, the PFS was highly correlated with the platelet function measurements used to construct the PFS, with the weakest correlation with PFA100 and strongest with epinephrine LTA. (Table 3) To validate the PFS we assessed the correlation between PFS and platelet function measures in HV2 and DM cohorts. In these cohorts, the PFS was calculated by taking their platelet function measures and applying the weights derived from the PCA from HV1. In these validation cohorts, the PFS significantly and strongly correlated with each measure of NCDPF, with a similar strength and direction as in HV1 (Table 3).

Acute effects of aspirin on COX-1 dependent platelet function

COX-1 dependent platelet function, as assessed by AAA, was effectively suppressed by a single, 325mg dose of aspirin in all HV2 and DM subjects; the *in vitro* addition of aspirin had no further effect on AAA. (Figure 2)

Acute effects and characteristics of aspirin on NCDPF

The main measure of NCDPF in this study, the PFS, was sensitive to the influence of aspirin as demonstrated by a significant shift in the PFS distribution to lower values (Figure 3): mean difference in Pre vs. Post PFS, [95% confidence interval] for HV1, HV2, and DM: 3.4 [3.0–3.7], 4.8 [4.5–5.2], 4.2 [3.6–4.8], paired t-test p-value < 0.0001 for all comparisons. In contrast to the uniform suppression of COX-1 dependent platelet function (Figure 2) after a single 325mg dose of aspirin, NCDPF persisted and demonstrated wide interindividual variation (Figure 3). We also found a significant, and strong correlation between the Pre and Post PFS in all three cohorts (Figure 4, $r = 0.77$, 0.81 , and 0.73 for HV1, HV2, and DM cohorts, respectively; $p < 0.0001$ for all).

In healthy volunteers, daily aspirin therapy results in a time-dependent shift towards higher NCDPF

After two weeks of aspirin in HV1, we observed a strong correlation between the Post and Final PFS values ($r = 0.74$, $p < 0.0001$). Despite this high degree of correlation, we observed that the PFS measured after two weeks of aspirin was shifted higher (mean difference Post and Final PFS = 0.5 , $[0.1–0.8]$, paired t-test $p = 0.01$) towards pre-aspirin levels of platelet function. In the HV2 cohort we also observed a similar shift to higher PFS after four weeks of aspirin (Figure 5A, mean difference between Post and Final PFS = 0.7 , $[0.2–1.2]$, paired t-test $p = 0.004$). This shift towards higher PFS over time was accompanied by a similar

trend in most but not all of the individual components of the PFS (epinephrine: paired t-test p-values: 0.05 for 10 and 1 μ M in HV1 and < 0.008 for all concentrations in HV2; ADP: $p = 0.07$ for 10 μ M in HV1, $p = 0.03$ for 5 μ M in HV2; Collagen: $p < 0.005$ for 2 μ M in HV1 and $p < 0.07$ for all concentrations in HV2; PFA100: $p = 0.01$ for HV1). These observations demonstrate that there was a significant change in NCDPF induced by aspirin in healthy volunteers.

Heterogeneity in the change in NCDPF during aspirin exposure depends on initial response to aspirin

Although *on average* PFS increased during aspirin exposure in both groups of healthy volunteers, on inspection of the individual trends in HV2 (Figure 5A) it was apparent that there was heterogeneity in these trends with some trending to higher Final PFS and others trending to lower Final PFS values. Stratifying the HV2 cohort based on those that increased their Final PFS (Figure 5B) vs. those that decreased their Final PFS (Figure 5C) demonstrated that the Post PFS was significantly different (Post PFS in those that increased vs. decreased PFS: -1.0 vs. 1.68 , t-test $p = 0.002$) between groups. To better characterize this heterogeneity, we correlated the change in PFS (Final - Post) and the Post PFS in the HV1 cohort and found an inverse correlation ($r = -0.45$, $p = 0.001$, Figure 6A), suggesting that the basis for the direction and magnitude of change in PFS during aspirin therapy was the initial response to aspirin. To validate this observation, we hypothesized that we would find an inverse correlation in HV2 and DM and again found a pattern in the same direction and with a greater strength as in the HV1 cohort (HV2: $r = -0.54$, $p < 0.0001$, Figure 6B; DM: $r = -0.68$, $p < 0.0001$, Figure 6C). Combining data from all three cohorts, the PFS after the first dose of aspirin was significantly correlated with the change in PFS during aspirin therapy ($r = -0.59$, p -value < 0.0001 , Figure 6D).

The increase in NCDPF induced by aspirin is not due to uninhibited COX-1

In the HV2 and DM subjects, we found that AAA (median % aggregation = 3.0, range: 0–7%) and in HV2 serum thromboxane B2 concentration (mean concentration = 0.3, range 0.1–2.0 ng/ml) remained suppressed and did not correlate with the Final PFS ($r < 0.2$, $p > 0.3$).

To demonstrate that the change in NCDPF induced by aspirin is not due to uninhibited COX1, in a subset of subjects ($n = 5$ HV2 and $n = 15$ DM subjects) we used the *in vitro* addition of aspirin to test if additional aspirin could further reduce NCDPF. We found that the *in vitro* addition of aspirin caused no further reduction in NCDPF using epinephrine 0.5 μ M (mean change aggregation with *in vitro* aspirin = 2.0%, $[-0.8 - 4.8\%]$, paired t-test $p = 0.2$), ADP 5 μ M (mean change = -2.3% $[-6.9 - 2.4\%]$, paired t-test $p = 0.3$), or collagen 2mg/ml (mean change = 1.5%, $[-6.9, 10.0]$, paired t-test $p = 0.71$).

Discussion

In this study, we utilized a variety of platelet agonists before and after the administration of aspirin in conjunction with a principal components analysis (PCA) to derive an integrated measure of non COX-1 dependent platelet function (NCDPF) in a cohort of healthy volunteers (HV1), which we term the platelet function score (PFS). We first validated the PFS in two additional cohorts (HV2 and DM) and then employed the PFS to characterize platelet function with aspirin therapy. The main findings of these investigations are 1) NCDPF test results are highly correlated and can be efficiently summarized into a single metric, the PFS; 2) despite complete suppression of COX-1 dependent platelet function with a 325mg aspirin dose, NCDPF persists and is characterized by wide interindividual variability that reflects pre-aspirin NCDPF; 3) compared to the response after the first dose,

daily aspirin therapy predictably results in one of two opposite effects despite continued suppression of COX-1 or the *in vitro* addition of aspirin: a) a return of NCDPF towards pre-aspirin levels or b) a continued decline in NCDPF. Based on these findings we conclude that the NCDPF response to aspirin is global and dynamic. Although our studies were not specifically designed to determine the mechanism(s) of this dynamic response, the overall findings suggest that changes over time may reflect an aspirin independent process.

The concept of a “global” platelet phenotype was first introduced by Yee and colleagues, in their description of a subset of individuals characterized by heightened platelet aggregation to submaximal doses of epinephrine.[3] These individuals demonstrated a robust aggregation response to not only epinephrine, but also other stimuli such as AA, collagen and ADP, as well as other aspects of platelet function such as adhesion and activation.[23] Our findings are consistent with those of Yee: We observed a correlation between all test results (Table 2), serving as the fundamental basis to construct the PFS. Our group was among the first [24] to use mathematical methods to summarize multiple measures of platelet function. Ohmori [25], Zufferey and colleagues[26] both utilized factorial analysis to identify a common “factor” that correlates with multiple measures of platelet function. Mathias and colleagues used PCA of platelet function measures in a genome-wide linkage study.[27] Our findings confirm those of prior studies as we identified a common factor, the PFS, which correlates with multiple measures of platelet function. A corollary to these observations is found in a recent genome- wide association study (GWAS) of platelet function where several genetic variants were associated with multiple measures of platelet function.[28] These GWAS findings suggest that common factors (i.e, genetic variants) may underlie the response to multiple forms of platelet stimulation. Finally, the strong correlation between pre- and post-aspirin PFS suggests that variable platelet function on aspirin is a reflection of underlying, baseline, platelet function. We believe that our ability to identify a common factor, the PFS, that represents multiple measures of NCDPF strengthens and extends the concept[3] of a global platelet function phenotype.

Our finding that PFS on aspirin does not correlate with AAA or serum thromboxane B2 further strengthens the hypothesis that the response to aspirin should be considered from two related but fundamentally distinct perspectives: COX-1 dependent (exemplified by AAA and serum thromboxane B2) and non COX-dependent (such as ADP, collagen, and epinephrine induced aggregation) platelet function. Prior studies[13,8,25], have shown that persistent NCDPF on aspirin is not due to incomplete COX-1 inhibition. Our data highlights that while COX-1 dependent platelet function can be suppressed by 325mg/day aspirin, NCDPF on aspirin cannot, and instead may require alternate or additional antiplatelet therapy. Last, we conclude that the true laboratory “response” to aspirin cannot be based on a COX-1 dependent test but also requires measures of NCDPF. Accordingly and as demonstrated by Frelinger and colleagues, the true clinical response to aspirin (i.e, protection from cardiovascular events) is a function COX-1 dependent *and* non COX1 dependent platelet function in individuals using aspirin for cardioprotection[9].

In our comparisons of NCDPF response to aspirin after single vs. repeated doses we found a strong and significant correlation in the PFS. This is consistent with current thinking that pre-aspirin platelet function is reproducible within individuals[3] and extends this position further to the platelet function response to aspirin. Despite this high level of correlation, there was a shift towards higher PFS values after repeated doses of aspirin (Figure 5) in most healthy individuals. A simple explanation for such a shift would have been nonadherence with aspirin[29]. After a systematic search through the use of medication diaries and pill counts, we could not find any evidence for nonadherence. Further, we tested for nonadherence [30] using COX-1 dependent platelet function assays which showed complete inhibition of aggregation after repeated aspirin dosing. Most importantly, because

in vitro aspirin did not further reduce NCDPF, we feel confident that neither nonadherence nor unsuppressed COX-1 activity were the causes for the observed changes in NCDPF. Although the mechanism for this shift in NCDPF is not yet known, it was seen across all agonists and most apparent using epinephrine as an agonist, a finding which suggests that epinephrine-induced aggregation is a marker of global platelet function[3,5,14]. Our study adds a dimension to prior observations with low dose aspirin by demonstrating that decreasing response over time in some subjects occurs despite the use of high dose aspirin and cannot be overcome *in vitro* even with high concentrations of aspirin [18]. Accordingly, although NCDPF can be used to measure the effect of aspirin, we believe the observed changes in NCDPF over time are due to an aspirin insensitive mechanism.

The direction and magnitude of the change in platelet function is not constant, but instead, varies as a function of the initial response to aspirin. The consistent, strong, and inverse correlation between the change in PFS induced by aspirin (Figure 6D) demonstrates that for a given subject, NCDPF is regulated and can respond to drug therapy in a time- and platelet function dependent manner. To illustrate the magnitude of the changes in NCDPF over time, for each unit change in PFS, there is a 7% difference in the maximal aggregation with 10uM epinephrine, a 3% difference with 10uM ADP, and a 5% difference with 5mg/ml collagen. Since the magnitude of change in PFS ranged from -5.0 to 5.0 (i.e. y-axis in Figure 6), changes in NCDPF, in some subjects, may lead to a clinically important difference in overall platelet function. Although the mechanism is unknown, possible explanations include (1) aspirin having a direct effect on the megakaryocyte or on a feedback loop between circulating platelets and megakaryocytes to regulate the level of overall platelet function;(2) a homeostatic mechanism to restore platelet function during antiplatelet therapy in healthy individuals, while for those with heightened platelet function[3] there may be a “reserve” of platelet function that can be further inhibited with prolonged aspirin therapy. Consistent with the latter hypothesis, a majority (67%) of diabetics in our study - a group characterized by heightened platelet function[31] - demonstrated a decrease in NCDPF during prolonged aspirin therapy. Therefore in individuals with heightened platelet function, whether due to an inherent difference[3] or a diseased state such as diabetes, aspirin induces an initial, acute decline in NCDPF followed by a continued slower decline over time. In contrast those with low/normal levels of NCDPF also experience an acute decline; however, their platelet function is characterized by a return towards pre-aspirin levels over time.

There are several limitations of our study that deserve consideration. First, because our study design focused on subjects in the upper/lower quartiles of NCDPF in HV2 and DM after the first dose of aspirin, the observations may be due to a “regression to the mean” phenomenon. However, in the HV1 cohort we did not focus on the extremes of the platelet function distribution and instead took an unselected sample. Therefore regression to the mean may account for the greater magnitude of correlation in the HV2 and DM cohorts compared to HV1, however, it is unlikely to explain the overall observation seen in all three cohorts. A true test would be to compare single-dose responses in NCDPF to aspirin separated by several weeks with the multi-dose data presented in these studies. Second, because we performed a large number of LTA measurements, the PFS may be biased towards these measures over the PFA100. The correlation between PFS and PFA100 was relatively weak and is consistent with a potential bias towards LTA measurements. Alternatively, since there are many other factors (e.g. von Willebrand factor, platelet count, and hematocrit [32]) that also influence PFA100 test results, the PFS may only capture that component of the PFA100 test that relates to platelet function. Finally, we did not assess COX-1 dependent platelet function in HV1; however, we did measure COX-1 dependent platelet function with AAA and/or assayed serum thromboxane in the HV2 and DM cohorts.

In summary, non COX-1 dependent platelet function is a global platelet phenotype that can be summarized using the PFS. The PFS in our studies of healthy volunteers and diabetics enable us to conclude that the platelet function response to aspirin is dynamic and should be based on non-COX1 dependent measures of platelet function made after several weeks of daily aspirin therapy. Future studies investigating the mechanisms of this response may uncover novel molecular pathways that are critical for the regulation of platelet function and the response to antiplatelet therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was funded by institutional funds provided by the Duke Institute for Genome Sciences & Policy, a National Institutes of Health (NIH) T32 Training grant (5T32HL007101 to DV), a grant (5UL1RR024128) from the National Center for Research Resources (NCRR), a component of the NIH, and NIH Roadmap for Medical Research, a grant (5RC1GM091083 to GSG) from the National Institutes of General Medical Sciences, and a grant (5U01DD000014-06 to TLO) from the Centers for Disease Control and Prevention. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official view of NCRR or NIH.

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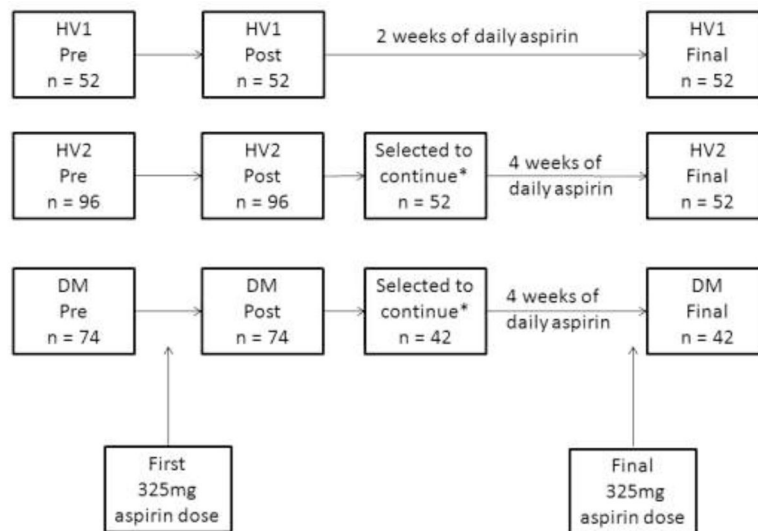


Fig. 1. Study overview

An overview study flow for the two healthy volunteer cohorts (HV1 and HV2) and diabetic cohort (DM). All subjects had platelet function measures (detailed in Methods) made before (Pre) and 3 hours after (Post). All HV1 subjects and selected HV2 and DM subjects continued with daily aspirin therapy and returned for a Final assessment of platelet function made 3–5 hours after the final aspirin dose. * = see selection protocol in Methods

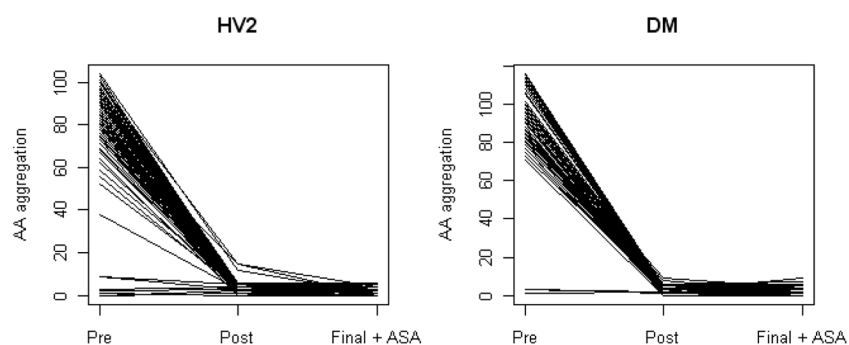


Fig. 2. COX-1 dependent platelet function with aspirin

Arachidonic acid-induced platelet aggregation before (Pre), 3 hours after 325mg aspirin by mouth without *in vitro* aspirin (Post), and 3 hours after 325mg aspirin by mouth with *in vitro* aspirin (Post + ASA) % aggregation on Y axis; AA arachidonic acid concentration = 0.5mM; *in vitro* aspirin concentration = 53 uM

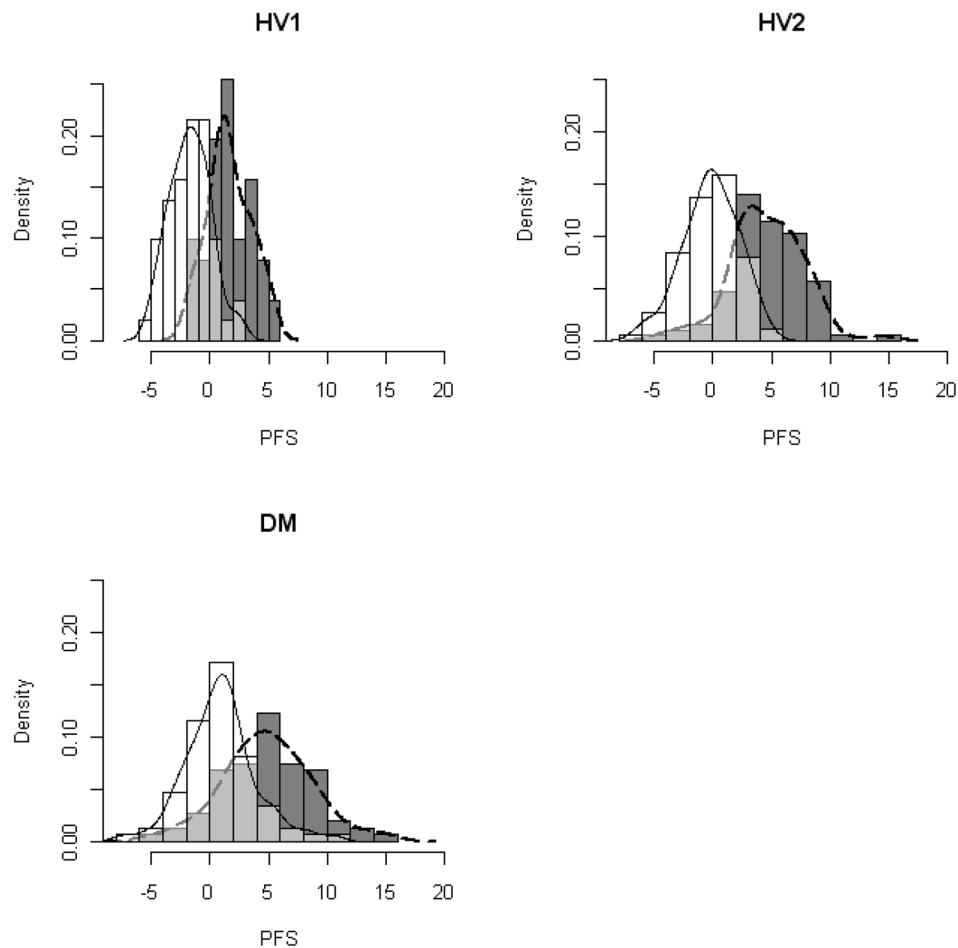


Fig. 3. Distributions of Platelet Function Score (PFS)

Histograms of platelet function score (PFS) for two cohorts of healthy volunteers (HV1 and HV2) and a cohort of diabetics (DM), before (Pre, shaded) and 3 hours after a single 325mg aspirin dose (Post, unshaded). The curves represent the probability density functions for the Pre (dashed) and Post (solid) PFS distributions, demonstrate the shift towards lower platelet function with a single dose of aspirin, and that variable pre-aspirin platelet function is largely retained post-aspirin

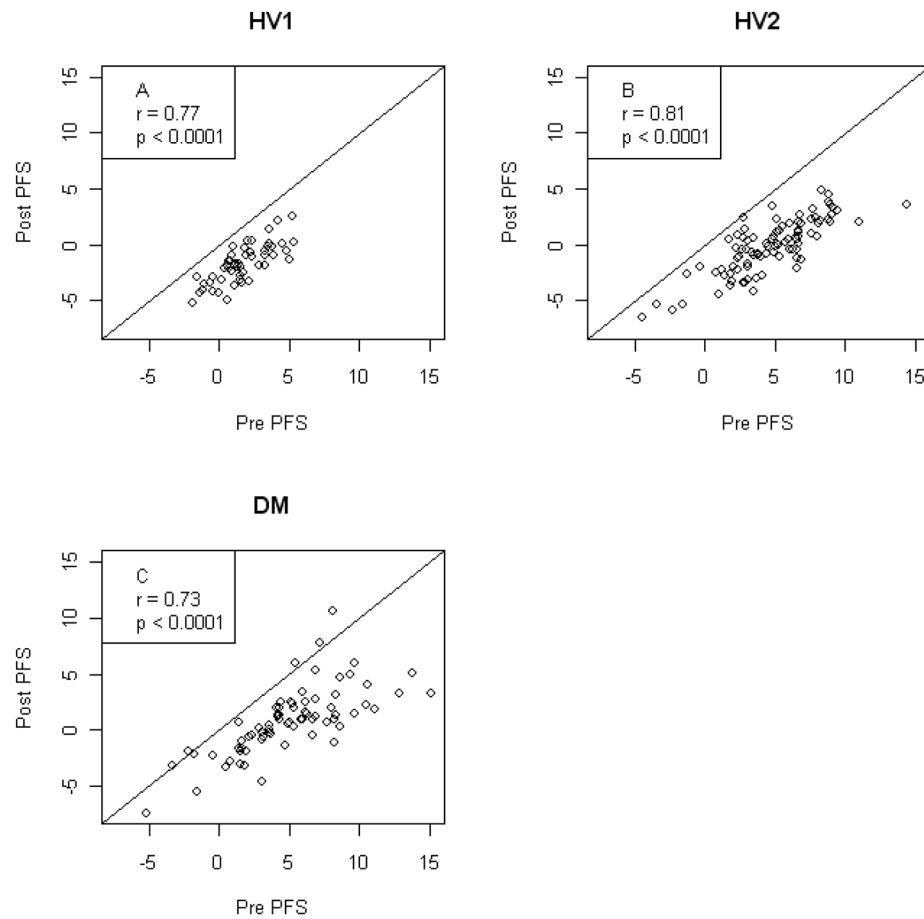


Fig. 4. Pre aspirin PFS predicts Post aspirin PFS

Relationship between non COX-1 dependent platelet function measured pre-aspirin (Pre PFS, on x-axis) and the immediately after the first 325mg aspirin dose (Post PFS, on y-axis) in two cohorts of healthy volunteers (A and B) and diabetics (C). Although there is a strong correlation between pre- and post-PFS in each cohort, the effect of aspirin in each cohort is demonstrated by individual points lying below the solid line (slope = 1, intercept = 0)

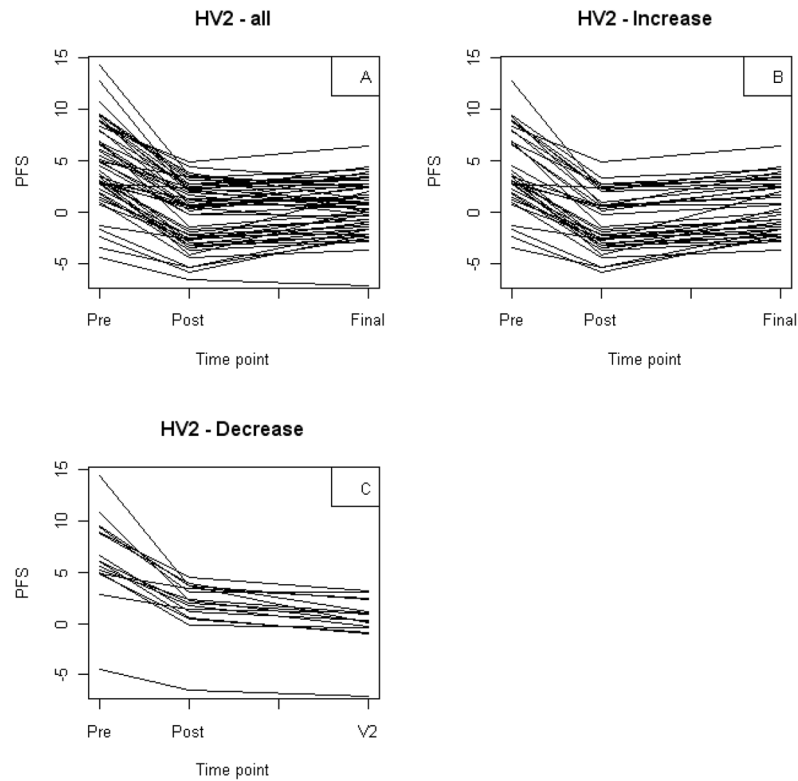


Fig. 5. Temporal changes in PFS during daily aspirin therapy

Non COX1 dependent platelet function as assessed by PFS at three time points in HV2 cohort (A): pre-aspirin (Pre), after the first 325mg dose of aspirin (Post), and after 4 weeks of 325mg/day aspirin (Final). The HV2 cohort was then divided based on an increase (B) or decrease (C) in PFS over time.

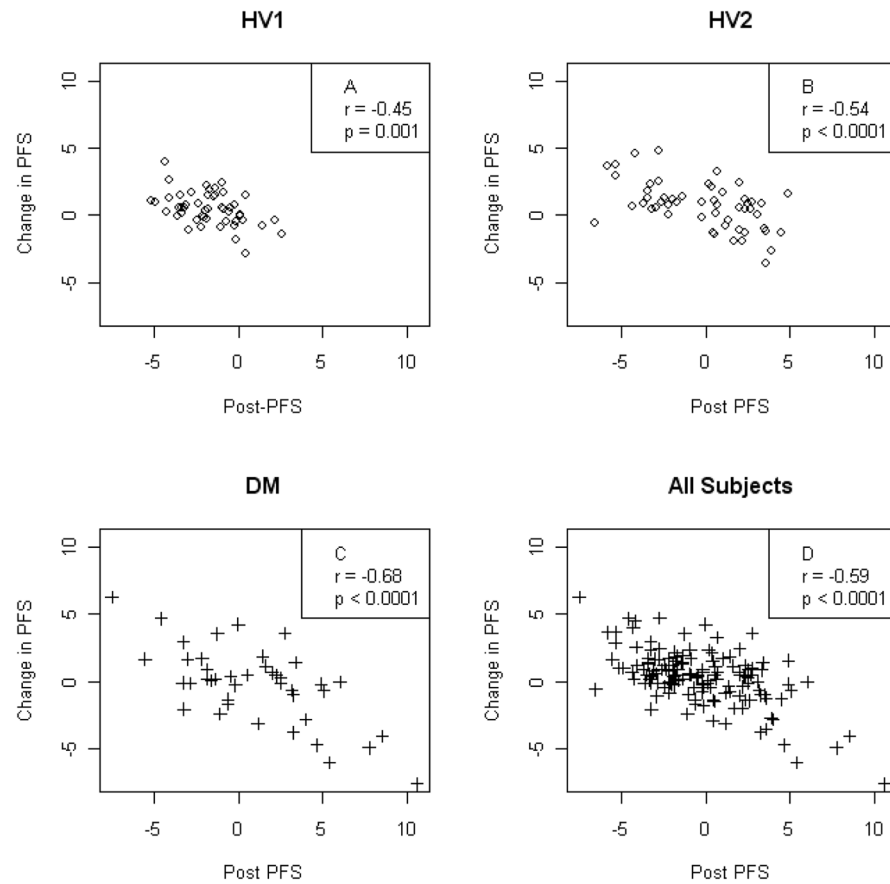


Fig. 6. Time-dependent change in PFS on aspirin is a function of acute response

Change in platelet function score (PFS) defined as Final - Post PFS (y-axis) vs. Post PFS (x-axis) in HV1 cohort (A), HV2 cohort (B), diabetics (DM, C), and in all subjects (D). Open circles = healthy volunteers. Crosses = diabetics. The magnitude and direction of the change in PFS over time is inversely proportional to the initial response to aspirin in each cohorts

Table 1

Baseline characteristics

	HV1 (n= 52)	HV2 (n = 96)	DM (n =74)
Age (mean +/- SD, years)	31 ± 9	43 ± 9 *	55 ± 11 *
Female (n)	26	59	46
Race (n, white/black/other)	36/9/7	60/32/4	40/30/4
Medications (n)			
• OCP	5	6	0
• Insulin	-	-	9
• Oral agents	-	-	59
• Diet control	-	-	6
• Anti-HTN	-	-	49
• Lipid lowering	-	-	37

* p < 0.001 for comparison with HV1; OCP = oral contraceptive pills; HTN = hypertensive

Table 2
Significant correlations between measures of non-COX dependent platelet function in HV1.

	PFA	ADP 10 μ M	ADP 5 μ M	ADP 1 μ M	Epi 10 μ M	Epi 1 μ M	Epi .5 μ M	Col 5 μ g/ μ l	Col 2 μ g/ μ l
PFA		-0.25	-0.29	-0.27	-0.50	-0.50	-0.51	-0.23	-0.29
ADP 10 μ M	-0.25		0.87	0.61	0.65	0.58	0.58	0.79	0.74
ADP 5 μ M	-	0.83		0.65	0.65	0.58	0.62	0.77	0.67
ADP 1 μ M	-	0.42	0.54		0.66	0.66	0.70	0.53	0.58
Epi 10 μ M	-0.28	0.66	0.66	0.49		0.91	0.92	0.60	0.66
Epi 1 μ M	-0.26	0.47	0.52	0.61	0.79		0.94	0.53	0.65
Epi 0.5 μ M	-0.22	0.41	0.42	0.58	0.62	0.83		0.57	0.68
Col 5 μ g	-0.44	0.56	0.41	-	0.45	0.33	0.26		0.87
Col 2 μ g	-0.55	0.55	0.49	-	0.44	0.32	-	0.75	-

Significant ($p < 0.05$) Pearson correlation coefficients are reported before (below diagonal), after (above diagonal) aspirin. PFA = PFA100 closure time in seconds; Remaining measurements used area under the light transmittance aggregometry curve; Epi = epinephrine; Col = Collagen

Table 3

Significant correlations between measures of non-COX dependent platelet function and PFS in derivation (HV1) and validation (HV2 and DM) cohorts

Cohort	PFA	ADP 10 μ M	ADP 5 μ M	ADP 1 μ M	Epi 10 μ M	Epi 1 μ M	Epi .5 μ M	Col 5 μ g/ μ l	Col 2 μ g/ μ l
Correlation with Pre PFS									
HV1		0.72**	0.79**	0.69**	0.88**	0.87**	0.78**	0.55**	0.68**
HV2		0.83**	0.82**	0.62**	0.80**	0.85**	0.79**	0.57**	0.74**
DM	-0.21**	0.87**	0.84**	0.81**	0.82**	0.80**	0.77**	0.69**	0.84**
Correlation with Post PFS									
HV1	-0.48**	0.89**	0.89**	0.81**	0.89**	0.89**	0.85**	0.89**	0.88**
HV2	-0.34*	0.85**	0.89**	0.75**	0.77**	0.73**	0.74**	0.80**	0.75**
DM	-0.27*	0.87**	0.87**	0.83**	0.75**	0.71**	0.76**	0.85**	0.80**
Correlation with FinalPFS									
HV1	-0.55**	0.85**	0.85**	0.72**	0.83**	0.79**	0.84**	0.87**	0.91**
HV2	-0.52**	0.81**	0.89**	0.62**	0.80**	0.80**	0.81**	0.85**	0.84**
DM	-0.45*	0.87**	0.88**	0.76**	0.82**	0.76**	0.77**	0.85**	0.78**

The platelet function score (PFS) was derived in the HV1 cohort and validated in the HV2 and DM cohorts(as described in Methods). Significant correlations between PFS and each measure of platelet function; PFA= PFA100 closure time in seconds; Remaining measurements used area under the light transmittance aggregometry curve; Epi = epinephrine; Col = Collagen;

* p = 0.01 > p > 0.001;

** p <0.0001